A COMPARATIVE STUDY OF STANDARD METHODS AND ORGAN CULTURE FOR THE ISOLATION OF RESPIRATORY VIRUSES

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THE introduction of organ cultures of human ciliated epithelium has increased the proportion of cases of acute respiratory infection in which a virological diagnosis can be made (Higgins, 1966; Tyrrell and Bynoe, 1966). The improved isolation rates resulting from the use of this technique were, however, mainly due to an increased efficiency in the isolation of rhinoviruses.

Tyrrell and Bynoe (1966) had confined their examination to a small number of specimens from colds and had shown that a number of viruses, which at that time were detectable only by the inoculation of volunteers, could be grown in organ cultures. Higgins studied a somewhat larger number of cases of all types of acute respiratory infection, but all the specimens had been frozen prior to examination. Nevertheless, a number of viruses undetected on direct inoculation of tissue culture were isolated after passage in organ culture.

The aim of our present investigation was to compare the standard methods of virus isolation with the same methods after the specimen had been passaged in organ culture. Unfrozen specimens were used in this study so that the more labile viruses, such as respiratory syncytial virus and the parainfluenza viruses, could be detected in addition to other respiratory viruses.

MATERIALS AND METHODS

Samples

Specimens submitted for routine diagnosis were included in the study provided that they came from a case of acute respiratory illness and that organ cultures were available for their examination. The specimens consisted of nose and throat swabs conveyed in a transport medium and held on melting ice until inoculated into cultures. A dry throat swab was also taken for bacteriological examination.

Examination of specimens

Standard methods. The transport medium was inoculated, generally within 2 hr of collection of the swabs, into secondary cultures of monkey kidney, the Bristol line of HeLa and the WI₃₈ strain of human embryo diploid fibroblasts. Specimens were also examined in cultures of human embryo kidney and inoculated into suckling mice, usually after the specimen had been stored at -40°C . The dry throat swab was plated on blood agar, which was incubated aerobically at 37°C .

Organ cultures. Organ cultures were prepared from the nose and trachea of human embryos spontaneously or therapeutically aborted between the 12th and 26th wk of pregnancy. Cultures were inoculated with approximately 0.4 ml of the specimen and the

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organ culture fluids were harvested and replaced with fresh medium every other day for 9 or 10 days. The harvested fluids from each organ culture were pooled and held frozen at -40°C .

The frozen, pooled organ-culture fluid together with a convenient volume of the final unfrozen harvest was inoculated into cultures of monkey kidney, the Bristol line of HeLa and WI₃₈. The pooled organ-culture fluid was also inoculated into cultures of human embryo kidney when these were available. If no virus was detected by these examinations the pooled fluid from the original organ culture was passed in a further organ culture and the harvested fluids from these second organ cultures tested in tissue cultures; suckling mice were also inoculated.

Details of the collection and transport of specimens, the preparation and maintenance of tissue and organ cultures and the detection and identification of viruses have been described previously (Higgins, Ellis and Boston, 1963; Higgins, Boston and Ellis, 1964; Higgins).

RESULTS

During the 18 mth between May 1966 and November 1967, 176 of the 699 specimens received from cases of acute respiratory infection occurring in general practice were examined in parallel by the standard methods and by organ culture. Fifty-three of these specimens were from patients with colds, 33 from patients with a sore throat and 90 from cases of infection of the lower respiratory tract. A further 32 specimens from normal subjects or patients without respiratory disease were also examined in a similar manner.

TABLE I

Isolation of viruses and streptococci by standard methods and organ culture

Number of specimens	Agents isolated by both methods	Agents isolated only by standard methods	Agents isolated only by organ culture
176	22 Rhinovirus H type 4 Rhinovirus M type 2 Parainfluenza virus 3 1 Coxsackie virus B3 1 Poliovirus 1 1 Echovirus 9 1 Adenovirus 5	9 Rhinovirus H type 1 Rhinovirus M type 5 Herpes simplex virus 1 Coxsackie virus A10 1 Echovirus 22 2 Mumps virus 5 Streptococci Group A	8 Rhinovirus H type 1 Rhinovirus M type
Total 176	32 (18.2 per cent.)	24 (13.6 per cent.)	9 (5.1 per cent.)

Isolations in relation to the method employed

Sixty viruses and 5 strains of *Streptococcus pyogenes* were isolated from the 176 patients with respiratory infections and 2 Coxsackie A viruses from the 32 subjects without respiratory symptoms. The identity of the viruses isolated and the method by which they were detected are shown in table I. The commonest virus isolation was of a member of the rhinovirus group. No strain of influenza virus or respiratory syncytial virus was isolated and only 2 strains of parainfluenza virus were encountered. These findings were similar to those obtained by the study of the remaining 523 specimens in tissue culture alone, for among the viruses isolated only 10 strains of parainfluenza virus, 2 of

respiratory syncytial virus and none of influenza virus A or B were found. Twenty-six of the 45 rhinoviruses isolated were detected by both procedures, but the remaining 19 were isolated in almost equal numbers by only one or other of the two methods.

All the strains of herpes simplex virus, the enteroviruses and mumps virus detected were isolated in tissue culture, but only a proportion of these, all enteroviruses, were recovered from the organ-culture fluid. Similarly, the 2 Coxsackie A viruses isolated from the "controls" were isolated only by the standard methods.

*Effect of the method of isolation on the results obtained
in the detecting systems*

More detailed information about the sensitivity of the two methods may be obtained by comparing the number of systems in which the viruses were detected after direct inoculation of the specimen with those resulting from the inoculation of fluid from an organ culture previously exposed to the specimen.

TABLE II

Comparison of the isolation of enteroviruses in tissue culture and suckling mice directly from specimens and from the fluid of organ cultures into which the same specimens had been inoculated

Specimen no.	Virus isolated	Result of inoculating									
		specimen into					organ-culture fluid into				
		MK	HeLa	HEK	WI ₃₈	SM	MK	HeLa	HEK	WI ₃₈	SM
516/66	Polio 1 . . .	+	+	+	+	-	+	-	-	-	-
469/66	Coxsackie B3 .	+	+	-	-	-	-	+	-	-	-
481/66	Coxsackie A9 .	+	+	-	-	-	-	-	-	-	-
484/66	Coxsackie A10	-	-	-	-	+	-	-	-	-	-
524/66	Coxsackie A10	-	-	-	-	+	-	-	-	-	-
108/67	Echo 9 . . .	+	-	-	-	-	+	-	-	-	-
706/66	Echo 22 . . .	+	-	-	-	-	-	-	-	-	-

MK = Monkey kidney; HEK = human embryo kidney; WI₃₈ = human embryo fibroblast; SM = suckling mice. + = Virus isolated; - = no virus isolated.

Such a comparison for the enteroviruses is given in table II where it can be seen that not only did 4 of 7 strains fail to multiply in the organ culture, but in two further instances, poliovirus 1 and Coxsackie virus B3, the virus in the organ-culture fluid was detected in only one type of tissue culture though it gave rise to a cytopathic effect in two or more systems after direct inoculation of the specimen.

Although five strains of herpes simplex virus were detected by direct inoculation of tissue culture none was recovered from the respective organ-culture fluids and similar results were obtained for the 2 strains of mumps virus isolated.

TABLE III

Comparison of the isolation of rhinoviruses in tissue culture directly from specimens and after passage in organ cultures

(a) *Rhinovirus M type*

Specimen no.	Result of inoculating					
	specimen into			organ-culture fluid into		
	MK	HEK	WI ₃₈	MK	HEK	WI ₃₈
664/66	—	—	+	—	+	+
665/66	—	—	—	—	—	+
366/67	+	—	—	+	—	—
468/67	+	—	+	—	+	+
897/67	—	—	+	+	+	+
1259/67	+	—	+	—	—	—

(b) *Rhinovirus H type*

Specimen no.	Result of inoculating				Specimen no.	Result of inoculating			
	specimen into		organ-culture fluid into			specimen into		organ-culture fluid into	
	HEK	WI ₃₈	HEK	WI ₃₈		HEK	WI ₃₈	HEK	WI ₃₈
306/66	+	+	+	+	331/67	—	+	+	—
210/67	+	+	+	+	379/66	—	—	—	+
519/67	+	+	+	+	437/66	—	—	—	+
243/66	+	+	—	+	909/66	—	—	—	+
2/67	+	+	—	+	6/67	—	—	—	+
998/67	+	+	+	—	541/67	—	—	—	+
1001/67	+	+	+	—	1031/67	+	—	—	+
995/67	+	+	—	—	732/66	+	—	+	—
525/66	—	—	+	+	745/67	+	—	+	—
331/66	—	—	+	+	1136/67	+	—	+	—
663/66	—	—	+	+	353/66	+	—	—	—
347/66	—	+	—	+	354/66	+	—	—	—
363/66	—	+	—	+	355/66	+	—	—	—
474/66	—	+	—	+	533/66	+	—	—	—
517/66	—	+	—	+	765/66	+	—	—	—
766/66	—	+	—	+	777/67	+	—	—	—
506/67	—	+	—	+	810/67	+	—	—	—
557/67	—	+	—	+	1053/67	+	—	—	—
1023/67	—	+	—	+	435/66	—	—	+	—
1090/67	—	+	—	+					

MK = Monkey kidney; HEK = human embryo kidney; WI₃₈ = human embryo fibroblasts.
 + = Virus isolated; — = no virus isolated.

Three tissue-culture systems were employed for the detection of the rhinoviruses: monkey kidney, which was applicable only to the M strains, human embryo kidney and WI₃₈ for both M and H types of rhinovirus. The manner in which the 45 rhinoviruses were isolated in these tissue culture systems by the direct inoculation of specimens and from the organ-culture fluids is shown in table III. Of the 45 strains, 16 behaved in an identical manner in tissue culture irrespective of the method of isolation. Ten rhinoviruses (9 H and 1 M type) were isolated by direct inoculation of the specimen into tissue culture, but could not be recovered from the organ-culture fluid. However, 8 of these strains were detected only in cultures of human embryo kidney. Nine viruses, 8 H type and 1 M, were detected only from organ-culture fluid and 6 of these were detected only in cultures of human embryo diploid fibroblasts. Of these 9 viruses isolated only in organ culture, typing has been attempted on 5, of which one was not neutralised by any of the 22 sera available and the remaining 4 were shown to belong to different serotypes (one each of type 2, type 6, type 15 and type 26).

The 32 persons without respiratory infections yielded one Coxsackie virus A10, which was isolated from a child who developed a cold 2 days after the specimen was collected, and one Coxsackie virus A9 from another child who suffered a rubella-like illness 9 days later and was still excreting the virus at that time.

DISCUSSION

The earlier findings in this laboratory (Higgins, 1966) and at Salisbury (Tyrrell and Bynoe, 1966), as well as the isolation of a new group of respiratory viruses (Tyrrell and Bynoe, 1965; McIntosh *et al.*, 1967; Tyrrell, Bynoe and Hoorn, 1968) suggested that organ culture was the more sensitive method for the investigation of acute respiratory infections. In the present study more agents were isolated by the standard methods than by organ culture. These agents were enteroviruses, herpes simplex virus and mumps virus and this is not unreasonable, for it is unlikely that the nose or the trachea is their site of multiplication in natural infections.

The position in respect of the rhinoviruses is not so clear. It should be noted that 8 of the 10 strains isolated by standard methods, but not by organ culture, were detected only in cultures of human embryo kidney. Different batches of these cultures are commonly employed in examining the original specimens and the organ culture fluids. Variation in the sensitivity of cultures derived from different embryos (Hobson and Schild, 1960; Johnson *et al.*, 1962; Higgins) may account for the differences observed between the two methods when the virus is isolated only in human embryo kidney. A similar variation in the susceptibility of the organ cultures derived from different embryos may also exist and has already been postulated to account for the inconsistent results obtained when attempts are made to grow the MR virus in organ culture (Tyrrell *et al.*) A number of rhinoviruses isolated only in organ culture have been shown to belong to serotypes previously isolated in tissue culture (Higgins; Tyrrell *et al.*) and additional strains are reported in this

study. The results obtained do not support the view that the efficiency of organ cultures in isolating rhinoviruses is due to an increase of the quantity of virus inoculated into tissue culture.

The over-all isolation rate (37 per cent.) obtained in the present study is not much higher than that generally obtained by standard methods alone. However, in another study during the same period, in which the same tissue- and organ-culture methods were used, 32 agents were isolated from 39 specimens, suggesting that the illnesses sampled during the present investigation were mainly due to agents that could not be detected by the methods employed. Although no appreciable diminution in activity of the cilia was observed it is possible that these specimens contained the more fastidious type of rhinoviruses, e.g., HS (Hoorn and Tyrrell, 1966) or ST strains (Tyrrell *et al.*) or the ether-labile avian infectious bronchitis-like viruses.

Specimens that were negative on examination by the standard methods failed to yield viruses (other than rhinoviruses) by organ-culture techniques as well. It is possible, however, that the standard methods are inefficient in isolating some strains of parainfluenza viruses (Tyrrell *et al.*). Unfortunately it was not possible to compare the efficiency of the two methods in isolating the more labile respiratory viruses, for there were few or no infections with influenza, parainfluenza or respiratory syncytial viruses during the period of the study. From the practical point of view it would appear to be wasteful to inoculate all unfrozen specimens into organ culture. It is better to conserve the foetal material for the examination of those specimens that fail to yield an agent by the standard methods.

SUMMARY

Fresh unfrozen specimens from 176 cases of acute respiratory infection and from 32 normal subjects, or patients without respiratory symptoms, were tested in parallel by direct inoculation into tissue cultures and suckling mice and also after the specimen had been passed in two organ cultures of human embryonic ciliated epithelium. Thirty-two agents were isolated by both methods: 26 only by the direct inoculation of tissue cultures and suckling mice, and 9 only after passage in organ culture.

Enteroviruses, herpes simplex virus and mumps virus were more readily isolated directly from the specimens than from the corresponding organ-culture fluid.

The only viruses isolated from the organ-culture fluids and not directly from the specimens were rhinoviruses. However, an almost equal number of these viruses were isolated directly from the specimens but not from the organ-culture fluid. It is postulated that these differences reflect the variation in the sensitivity to rhinoviruses of both the organ cultures and the kidney tissue cultures derived from different human embryos.

It is suggested that, in view of the limited supply of foetal material, it is uneconomical to test unfrozen specimens from respiratory infections in organ culture. This approach should be restricted to the examination of those

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